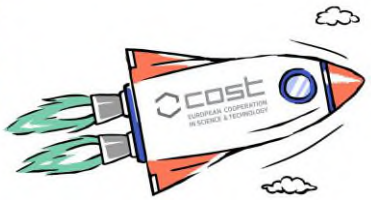


COST Action CA21115

Report date: November 2024

WG1. FeS Clusters and the Immune System

General description: This report describes the protocols developed and used by Action members for elucidating iron metabolism and iron-sulfur proteins in the immune system

Period: Nov 2022 – Nov 2024

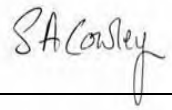
Section 1. Protocols and methods related to use of iPSC in elucidating iron-sulfur proteins and iron metabolism in immune cells like macrophages or microglia

The protocols and methods described here were used by Ms. Lynne Faherty during her visit to Dr Sally Cowley lab at the University of Oxford.

Lynne Faherty, a Ph. D student in Suzanne Cloonan's lab applied for funding to visit the Cowley lab:CA21115 - Short Term Scientific Mission titled Investigating the role of mitochondrial iron deficiency on alveolar macrophage metabolism and function. She was successful in this application. She was hosted by the Cowley lab for 3 weeks in July 2023, where she learnt to differentiate iPSC-to macrophages and to type 2 alveolar epithelial cells. She then cocultured the two cell types together at air-liquid interface and drafted a protocol for this. There is some further optimisation needed to improve the enrichment for lung epithelial cells , and to optimise the coculture conditions on air-liquid interface, but this has been a very beneficial scientific exchange.

Further to Lynne's visit, we have initiated an Material Transfer Agreement to be able to send one of our iPSC lines from Oxford to the Cloonan lab in Dublin for them to be able to set up co-cultures in their lab. This iPSC line constitutively expresses Red Fluorescent Protein at high levels in iPS-macrophages, making it an excellent choice for live imaging of iPS-macrophages in coculture with iPS-alveolar cells. The MTA has been signed now by both institutions, and shipping of the cells is currently being arranged. These effort all contribute to the establishment of the iPS systmes in the Cloonan lab to be able to explore iron metabolism in these cell types.

Standard Operating Procedure	JMSCFSOP04v2.6
Human iPS Cell Macrophage Differentiation via Embryoid Body formation using AggreWells	

Approval		Date Implemented: 09/04/2019	
	Name	Signature	Date
Author	Jane Vowles Cathy Browne Sally Cowley		10.04.2019
User Review	*Sign off on behalf of person/s reviewing the draft document*		10.04.2019

Change History Version No/Date Issued	Reason for Issue/Change Summary
V2 12.06.2016	V1 06-2013 updated to include scale-up of factories
V2.1 08.2016	V2 renamed, and made consistent with alternative procedure: JMSCF iPS-Macrophage Differentiation via Embryoid Body formation using Mechanical Dissociation v2.1 08.2016. More notes, troubleshooting and references added
V2.2 07.2017	V2.1 updated to be include reference to and be consistent with iPSC-microglia protocol – reference included, macrophage precursors as a starting point for microglia explained V2.2 updated to expand EB phase, updated related docs and product numbers, updated AggreWell preparation, added Expected Results section including photos, and minor edits throughout.

V2.3 08.2018	V2.3 updated to give new XVIVO and other product codes
V2.4 10.2018	V2.4 updated, general editing, formatting and streamlining of text Incorporation of more troubleshooting/efficiency improvements
V2.5 04.2019	
V2.6 09.2024	

1.0 Purpose

To produce embryoid bodies of uniform size/cell number using AggreWell™800 (300 microwells) from human Pluripotent Stem Cells which have been grown feeder-free on Geltrex or Matrigel, and differentiate them to macrophages

2.0 Scope

2.1 Harvesting a single cell suspension of iPS cells

2.2 Producing uniform embryoid bodies of known cell number for further differentiation to macrophages

2.3 Differentiation in 'Factories' to macrophage precursors

2.4 Further differentiation of precursors into macrophages

3.0 Related Documents

3.1 van Wilgenburg, B., Browne, C., Vowles, J., and Cowley, S. A. (2013). Efficient, long term production of monocyte-derived macrophages from human pluripotent stem cells under partly-defined and fully-defined conditions. PloS one, 8(8):e71098+.

3.2 Haenseler, W., Sansom, S. N., Buchrieser, J., Newey, S. E., Moore, C. S., Nicholls, F. J., Chintawar, S., Schnell, C., Antel, J. P., Allen, N. D., Cader, M. Z., Wade-Martins, R., James, W. S., and Cowley, S. A. (2017). A highly efficient human pluripotent stem cell microglia model displays a Neuronal-Co-culture-Specific expression profile and inflammatory response. *Stem cell reports*, 8(6):1727-1742.

3.3 JMSCFSOP19 Culture of Human iPS Cells from cryopreserved samples and expansion to create Masterstock v2.5

3.4 JMSCFSOP Differentiation to human iPS Cell-Microglia by iPS cell-Macrophage-Neuronal co-culture v2

3.5 STEMCELL Technical Manual: Reproducible and Uniform Embryoid Bodies Using AggreWell™ Plates

3.6 JMSCFSOP03 OXE8 medium preparation

4.0 Responsibility

5.0 Health Safety & Environment

Local, national and EU Health and Safety regulations must be adhered to. Human iPSC are to be handled within a Class II safety cabinet to protect the worker from possible adventitious agents. The patient should have been tested and be negative for HIV, HBV and HCV, but pathogen screening will never be comprehensive. Local, national and EU health and safety regulations must be adhered to.

6.0 Materials & Equipment

Product name	Supplier	Catalogue number
mTeSR™1 Medium	Stemcell Technologies	85850
Alternative to mTeSR™1: OXE8	Refer to JMSCF SOP_03	
Geltrex (hESC qualified, growth factor-reduced)	Life Technologies	A1413302
Alternative to Geltrex: Matrigel	Corning	15140-122
Penicillin-Streptomycin (P/S 100x)	Gibco	15140-122
TrypLE Express	Life Technologies	12604-013
Y-27632 (ROCKi)	AbCam Biochemicals	ab120129
PBS	Sigma	D8537-500ML
AggreWell™ 800	Stemcell Technologies	34811/34815
Aggrewell rinsing solution	Stemcell Technologies	7010
BMP-4	Invitrogen	PHC9534
VEGF	Invitrogen	PHC9394
SCF	Miltenyi	130-096-692
X-VIVO 15	SLS (Lonza)	BE02-060F
GlutaMAX (100x)	Gibco	35050-038
2-Mercaptoethanol (1000x)	Gibco	31350-010
IL-3	Invitrogen	PHC0033
M-CSF	Invitrogen	PHC9501
Cell strainer 40 µm	Falcon	352340
Alternative: 37µm Reversible strainers	Stemcell Technologies	27250
Low-attachment 6 well plates	Costar	3471
Alternative: Cellstar 6 well cell repellent surface	Greiner bio-one	657970

6.1 Reagent Prep

Growth factors

Make up according to manufacturer's instructions, store concentrated stocks frozen in aliquots at -80°C

Y-27632 (ROCKi)

100x stock solution @ 1mM in PBS

Keep frozen in aliquots at -20° C

EB Medium

mTeSR/OXE8

BMP4 @ 50ng/ml

VEGF @ 50ng/ml

SCF @ 20 ng/ml

Factory Medium

500ml XVIVO15

5ml GlutaMax

5ml P/S (optional)

500ul 2-Mercaptoethanol

M-CSF @ 100 ng/ml

IL-3 @ 25 ng/ml

Macrophage Medium

500ml XVIVO15

5ml GlutaMax

5ml P/S (optional)

M-CSF @ 100 ng/ml

7.0 Procedure

7.1 Seeding cells to AggreWell Day 0

7.1.1 For each well of an AggreWell 800 plate, 4 x 10^6 human induced Pluripotent Stem Cells (iPSC) are required, equivalent to 2-3 just-confluent wells of a 6-well plate grown without feeders on Geltrex (or hESC-qualified Matrigel) in mTeSR1/OXE8

7.1.2 Add 0.5 ml AggreWell rinsing solution to well and centrifuge at 3000g for 3 mins to remove bubbles from microwells,

7.1.3 Aspirate, and rinse AggreWell with 1mL PBS

7.1.4 Aspirate PBS and add 1mL of 2X EB medium with ROCKi @ 10μM to each well, place in incubator whilst processing cells

7.1.5 Aspirate medium from iPSC and rinse with PBS

7.1.6 Aspirate PBS and add 1mL prewarmed TrypLE to each well, incubate at 37˚C for 5 minutes

7.1.7 Observe under microscope to check cells have detached and then dilute TrypLE 1:10 with PBS, collect in centrifuge tube

7.1.8 Ensure cells are well mixed and in single cell suspension by pipetting up and down 3-4 times very gently using a serological pipette

7.1.9 Take aliquot for cell count

7.1.10 Centrifuge at 400g for 5 minutes

7.1.11 After centrifugation and counting, aspirate PBS, tap cell pellet and resuspend in mTeSR/OXE8 medium with ROCKi @ 10μM to give a final cell concentration of 4 x 10^6/ml

7.1.12 Remove any obvious lumps

7.1.13 Add 1ml cells to the AggreWell (total volume in well 2ml) mix gently

7.1.14 Centrifuge plate 100g for 3 minutes

7.1.15 Examine AggreWell under the microscope to verify cells are evenly distributed among the microwells and not spilling over into adjacent wells

7.1.16 Very gently return AggreWell plate to back of incubator, making sure cells are not disturbed

NOTE 1: it is also possible to generate EBs by other spin methods, e.g. in 96-well low attachment plates, if required. See related SOP JMSCF: Production of Embryoid Bodies using 96-well low adherent plate Spin Method

7.2 Feeding Embryoid Bodies day 1-3

NOTE 1: Take care not to knock the plate as this will dislodge EBs from the microwells

NOTE 2: The EBs are kept in the AggreWell and fed daily as described below in order to prevent them adhering to each other, which may a problem if they are transferred to a non-adherent plate on day 2

7.2.1 Observe the plate under the microscope. One EB should be clearly visible in each microwell. There may also be some dead cells not incorporated into the EB

7.2.2 Bring EB medium to room temperature

7.2.3 Gently aspirate 1mL medium from top of AggreWell using a p1000 pipette tip
7.2.4 *Very gently* add 1mL fresh EB medium using a p1000 pipette tip, running slowly down the side of the well so the EBs are not washed out of the microwells
7.2.5 Repeat previous 2 steps to give a 75% medium change overall
7.2.6 Repeat daily for up to 3 days total in the AggreWell

7.3 Alternative feeding Embryoid bodies after day 2

7.3.1 Harvest EBs as described below in section 7.4. Using a 5 mL serological pipette, gently transfer the EBs into a well or 2 of a 6-well low-attachment plate with 4 mL EB medium per well
7.3.2 Move carefully to the incubator, ensuring that the EBs don't all swirl to the middle of the well (in which case they will merge together and downstream steps will be much less efficient)
7.3.3 Incubate for 2-3 days (over the weekend is ideal) without interference

NOTE 1: EBs need to have been exposed to EB medium for at least 4 – 6 days before setting up Factories. Different iPS lines may vary in the optimal timing for passing through mesoderm (BMP4) to hemogenic endothelium (VEGF/SCF). The exact number of days in the AggreWell versus low-attachment plates can be varied according to the day of the week of setup (it is convenient to setup AggreWells on Monday and move to low-attachment plates on Friday to avoid daily medium change).

7.4 Harvesting Embryoid Bodies from Aggrewells day 4

7.4.1 Place an inverted 40µm cell strainer on top of a centrifuge tube, wet with a few mL of PBS
7.4.2 Gently pipette the contents of the AggreWell up and down several times using a 5mL serological pipette (this has a wide enough bore not to damage the EBs) to dislodge the EBs from the microwells
7.4.3 Take up contents of well and transfer to the 40µm cell strainer taking care not to flood the strainer
7.4.4 Wash out the AggreWell several times with PBS, passing it through the strainer to wash the EBs on the strainer
7.4.5 Check the AggreWell under the microscope to ensure that all EBs have been removed
7.4.6 Turn the sieve over onto a new 50ml centrifuge tube and wash off the EBs into the tube using 5ml factory medium

7.5 Setting up Factories

7.5.1 Divide 300 EBs between 2 T175 flasks with 15mL factory medium in each, or if a smaller scale format is required (or if some EBs were lost during harvest) ~75 EBs per T75 flask in 8ml medium
7.5.2 Return flasks 'Factories' to the back of the incubator and don't be tempted to move them for a week

NOTE 1: the EBs do not distribute evenly in the pipette, so attention is required when distributing them between flasks. It is acceptable to put all 300 EBs into 1

T175 flask, but you will then have to feed more volume once the cells start to produce macrophage precursors.

7.6 Feeding Factories

7.6.1 Carry carefully, so as not to disturb any adhered EBs

7.6.2 If using T175 flasks, add 10ml factory medium per week or 5ml for T75 flasks, making sure the EBs are not flushed off the plastic

7.6.3 Continue to feed weekly with 10mL or more if medium becomes yellow

7.6.4 Once the Factories are producing good numbers of macrophage precursors, they can be harvested by taking the supernatant, always leave at least 20% of the medium in the factory, and then re-feed with fresh medium.

NOTE 1: Not all EBs will adhere to the plate so care has to be taken during feeding not to remove them

NOTE 2: Macrophage precursors usually start to be produced at around 4 weeks, depending on cell line. If correctly maintained, factories will continue production for several months and can be harvested weekly or fortnightly. Cells from the first harvests are usually still proliferative, so they are usually used as a pilot. Later harvests will not display post-harvest proliferation. Harvests 3-8 are usually very reliable as the Factories are in peak production. Subsequent harvests will produce smaller yields.

NOTE 3: It should be obvious when the macrophage precursors start to appear – previous to this there will be some debris and small dead cells in the supernatant, but the macrophage precursors are large (20µm diameter) and shiny and are generally round but with a few protrusions, phagocytic cups etc. They will clear up any debris, so the Factory will become much 'cleaner'

NOTE 4: Do not allow medium to become spent. This will hinder production of macrophages if at the start of differentiation, and at later stages, it will cause macrophages to die, and be eaten by others

NOTE 5: The floating cells produced at this stage are termed macrophage precursors. Previouly called 'Monocytes', because of their floating characteristic, and before understanding their ontogeny, but this differentiation pathway is now shown to be MYB-independent, ie to follow a primitive rather than adult, definitive myelopoietic programme (Buchreiser et al 2017) so it is not equivalent to a blood-derived monocyte, and in Hanseler et al 2017 is shown to separate out on PC1 and PC2 from blood monocytes.

7.7 Harvesting macrophage precursors and terminal differentiation to Macrophages

7.7.1 After 4-6 weeks numbers of floating macrophage precursors should be sufficient to harvest for macrophage differentiation or other experiments.

7.7.2 Gently harvest the supernatant, leaving at least 20mL medium being careful not to disrupt the Factory or floating EBs

7.7.3 Pass supernatant through a 40µm cell strainer to remove any large clumps or floating EBs which have been inadvertently removed from the Factory

NOTE 1: At this stage, cells can be transported to another lab, shipped overnight at ambient temperature, or left at 4°C for up to 48 hours. For transport/storage they should be centrifuged and resuspended at 4 x 10^6 /ml in cryovials, since is left in 50ml tubes, they will attach to the large surface area of plastic.
NOTE 2: Freezing/thawing has not been successful with these cells, so plan experiments to use fresh cells

7.7.4 Add warm factory medium to the Factory to replace the volume removed and return to incubator
7.7.5 Take an aliquot from harvested cells for cell count
7.7.6 Centrifuge at 400g for 5 minutes
7.7.7 Tap pellet and resuspend in macrophage differentiation medium to the required concentration. Seed at 0.3 x 10^6 per well of 24-well plate in 1ml medium (or equivalent density for other well-sizes)
7.7.8 Cells will begin to adhere within 24 hours, and a proportion become spindly over the course of the week (with some cell line variability)
7.7.9 At day 7 macrophages are ready to use
7.7.10 If the experiment extends longer, continue to change 50% medium every 4 -7 days

NOTE 1: Other macrophage differentiation media can be used. The XVIVO medium recommended here produces macrophages that have numerous large vesicles. Use of serum reduces the size/number of these vesicles. The macrophages can also be terminally differentiated with alternative factors to M-CSF if desired

NOTE 2: The macrophages are not very easy to replate, as they attach very strongly to tissue culture plastic and glass, so we recommend plating into the final format whenever possible

7.8 Troubleshooting

If no macrophage precursors appear in the Factories after 6 weeks, the most likely reason is a bottleneck in differentiation of earlier precursors in the EBs. To overcome this:

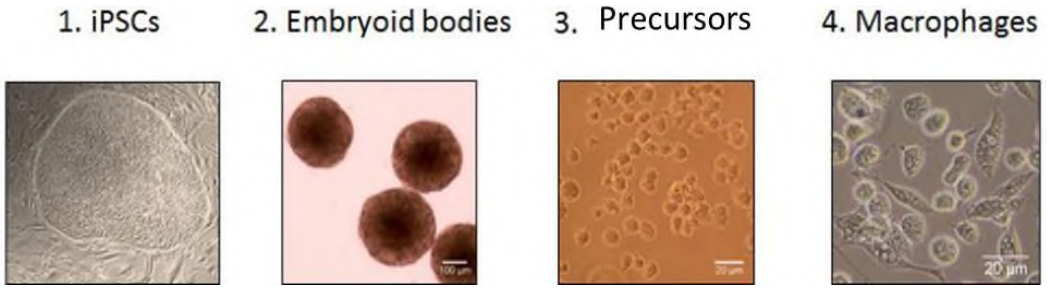
7.8.1 Adjust the number of days the EBs are kept in AggreWell and Low-Attachment plates
7.8.2 It is also possible to make AggreWell EBs from cells grown on MEFs, using this protocol – the MEFs do not interfere with AggreWell EB formation, and we find that subsequent macrophage yields are generally improved using cells that have been transferred onto MEFs one passage before the differentiation starting point
Other possible reasons:
7.8.3 EBs did not form properly, were too small, and/or disintegrated – make sure this stage is done quickly and with ROCKi, to ensure good viability

7.8.4 EB density too high or too low in the Factory. Too many EBs means the differentiation factors or nutrients become limiting. It is very important not to allow the medium to become spent.

7.8.5 Cell line is completely refractory to this differentiation pathway. This is very unusual, but is possible, in which case try other lines.

7.9 Expected results

Each T175 flask, containing 300 EBs at the start, should produce several million macrophage precursors per week, and where large numbers are needed at one time, it is possible to increase the medium in the T175 flask up to 150ml at the peak of production to harvest up to 30 million



7.10 References

Please cite the following paper from our lab in any publications that use this protocol, citing it in the main manuscript, not just in the supplementary information (where it would not be captured by citation managers):

van Wilgenburg, B., Browne, C., Vowles, J., and Cowley, S. A. (2013). Efficient, long term production of monocyte-derived macrophages from human pluripotent stem cells under partly-defined and fully-defined conditions. PloS one, 8(8):e71098+.

Other references utilising this method from our lab include:

Haenseler, W., Sansom, S. N., Buchrieser, J., Newey, S. E., Moore, C. S., Nicholls, F. J., Chintawar, S., Schnell, C., Antel, J. P., Allen, N. D., Cader, M. Z., Wade-Martins, R., James, W. S., and Cowley, S. A. (2017). A highly efficient human pluripotent stem cell microglia model displays a Neuronal-Co-culture-Specific expression profile and inflammatory response. *Stem cell reports*, 8(6):1727-1742.

Buchrieser, J., James, W., and Moore, M. D. (2017). Human induced pluripotent stem Cell-Derived macrophages share ontogeny with MYB-independent Tissue-Resident macrophages. *Stem cell reports*, 8(2):334-345.

van Wilgenburg, B., Moore, M. D., James, W. S., and Cowley, S. A. (2014). The productive entry pathway of HIV-1 in macrophages is dependent on endocytosis through lipid rafts containing CD4. PLoS ONE, 9(1):e86071+.

Flynn, R., Grundmann, A., Renz, P., Hänseler, W., James, W. S., Cowley, S. A., and Moore, M. D. (2015). CRISPR-mediated genotypic and phenotypic correction of a chronic granulomatous disease mutation in human iPS cells. Experimental hematology, 43(10).

This method builds on a previous method developed by our lab, which established the basic differentiation protocol but utilised serum-containing media, which led to less consistent differentiation:

Karlsson, K. R., Cowley, S., Martinez, F. O., Shaw, M., Minger, S. L., and James, W. (2008). Homogeneous monocytes and macrophages from human embryonic stem cells following coculture-free differentiation in M-CSF and IL-3. Experimental hematology, 36(9):1167-1175.

8.0 Definitions

XX

9.0 Troubleshooting

XX

10.0 Appendices

James Martin Stem Cell Facility 2014

CD14/CD45 antibody staining of Monocytes and Macrophages

Preparation of Macrophages

Day 0 **-** Harvest monocytes from factories (see SCL protocol - Production of monocytes and macrophages from iPS cells - 2013) and set up to differentiate to macrophages for 5 -7 days

Harvesting monocytes and macrophages for staining

Day 7 – Harvest macrophages Check under microscope that the monocytes have differentiated into macrophages

Aspirate medium from wells

Wash with PBS

Add 1ml cold Lidocaine/EDTA per well (of 6-well plate, or adjust volume for different plate formats)

Incubate on ice for 1 hour

After 1 hour, check under microscope (cells should have rounded up and be starting to lift off)

Gently pipette up and down with an eppendorf tip to remove cells from plastic, transfer to a centrifuge tube of PBS, wash out well with 1 - 2ml PBS, add to tube (check under microscope that cells have been lifted)

Record the volume and take an aliquot for cell count

Centrifuge at 400g for 5 mins

Remove and discard supernatant

Resuspend in FACS buffer to give approx 2 x 10^6/ml

Add 45µl cells to wells of a deep v-well microtitre plate (or use FACS tubes); one well for each antibody, another for the isotype control. (Will want to count live around 10^4 events so aim to plate at least 5 x 10^4 cells/well, ideally 10^5/well).

Harvest monocytes from factories (see SCL protocol - Production of monocytes and macrophages from iPS cells - 2013)

Centrifuge at 400g for 5 mins

Resusepend pellet in a known volume for PBS and take an aliquot for cell count

Centrifuge at 400g for 5 mins

Remove supernatant and resuspend in FACS buffer to give approx 2 x 10^6/ml

Add 45µl cells to wells of a deep v-well microtitre plate (or use FACS tubes); one well for each antibody, another for the isotype control.

Note: save a few unstained cells to calibrate FAC machine; wash in PBS and resuspend in a small volume FACS Fix

Note: CD14/CD45 use the same isotype control, so only need to set up 3 wells; 1 for each antibody, 1 for the isotype control

Antibody Staining

Add 5ul antibody or isotype (some may require diluting – see data sheet) to the cells, mix well
Note: it may save time to set up the plate with the antibodies/isotypes whilst incubating the macrophages with Lidocaine/EDTA, wrap in foil and store in the fridge until required

Incubate for at least 1 hour in the dark (wrap in foil), place on rocker at room temperature (or may incubate overnight at 4°C)

After 1 hour, wash cells by adding 200µl FACS buffer to each well, mix by pipetting up and down gently, centrifuge at 400g for 5 mins

Carefully aspirate 200µl supernatant, and repeat washing with 200ul FACS buffer, centrifuge at 400g for 5 mins

Carefully aspirate 200µl supernatant, then add 200 µl FACS FIX, mix gently

Store in dark at 4°C

Run through FACS within a week

Lidocaine/EDTA
5mM EDTA
12mM Lidocaine
Make up 500ml in PBS, store at 4°C

FACS Buffer
1% FCS (FBS)
10ug/ml IgG from human serum (stock at 1mg/ml - 100X)
0.01% Azide
Make up in 500ml PBS store at 4°C

FACS FIX
FACS buffer with 4% Formaldehyde
(Lab stock at 36%)

Antibodies
CD14 FITC ImmumoTools Product code: 21270143
CD45 FITC ImmunoTool Product code: 21270453
IgG1 FITC ImmumoTool Product code: 21335013

Human pluripotent stem cells derived Airway Epithelial Cells

Xu Liu*, Sai Liu†, William James‡, Sally Cowley§
March 8, 2023

1 Introduction

This protocol describes an approach to establish three-dimensional (3D) culture of PSC-derived EpCam^{+} alveolar cells (Fig.1), following Kotton's protocol published in Jacob et al. Nature Protocols (2019) and Hiemstra's protocol published in Sander et al. Sci Rep 10, 5499 (2020). More information can be found on our website.

2 Materials

2.1 Media components

(1) mTeSR^{TM}1

No.	Components	Cat.
1	mTeSR1 medium	StemCell Tech, 85850

(2) STEMdiff^{TM} Definitive Endoderm kit

No.	Components	Cat.
2	STEMdiff^{TM} kit	StemCell Tech, 05110

(3) cSFDM media

No.	Components	Cat.	Vol.	Stock Con.
3	cSFDM		(100mL)	
4	Iscoves modified Dulbecco's medium	Thermo, 21056-023	75mL	
5	Ham's F-12 Nutrient Mix	Thermo, 21765-029	25mL	
6	Pen Strep	Gibco, 15140-122	1mL	100x
7	Glutamax	Gibco, 35050-061	1mL	100x
8	Bovine Albumin Fraction (BSA)	Gibco, 15260-037	750µL	7.5%
9	B27	Gibco, 17504-044	1mL	100x
10	N2	Gibco, 17502-048	500µL	200x
11	Ascorbic acid	Sigma, A4544	100µL	50mg/mL
12	Monothioglycerol	Sigma, M6145	300µL	13µL in 1mL PBS

(4) DS/SB media

No.	Components	Cat.	Vol.	Stock Con.
13	DS/SB		(20mL)	
3	cSFDM		20mL	
14	SB43152	Sigma, S4317	1000x	10mM
15	Dorsomorphin	Sigma, P5499	1000x	2mM

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(5) CBRa lung progenitor–specification medium

No.	Components	Cat.	Vol.	Stock Con.
16	CBRA		(50mL)	
3	cSFDM		50mL	
17	CHIR99021	Tocris, 4423	50µL	3mM
18	rhBMP4	Sigma, P5499	50µL	10µg/mL
19	Retinoic acid	Sigma, R2625	50µL	100µM

(6) Alveolar differentiation CK+DCI medium

No.	Components	Cat.	Vol.	Stock Con.
20	CK+DCI media		(50mL)	
3	cSFDM		50mL	
17	CHIR99021	Tocris, 4423	50µL	3mM
21	cAMP	Sigma, B5386	50µL	100µM
22	IBMX	Mpbio, 195262	50µL	100µM
23	Dexamethasone	Sigma D4902	50µL	20mg/mL (50µM)
24	FGF-7 (rhKGF)	R&B, 251-KG-010	50µL	10µg/mL

2.2 Other reagents

- Dulbecco's PBS (no calcium, no magnesium; Gibco, cat. no. 14190144)
- TryplE (Gibco, cat. no. 12604013)
- Trypan blue (Invitrogen, cat. no. T10282)
- Matrigel (Corning, REF. no. 354277)
- CD326 (EpCAM) MicroBeads CD326 (MiltenyiBiotec, cat. no. 130-061-101)
- Dispase (Gibco, cat. no. 17105041)
- Freeze medium (For 10 ml, add 1 mL of DMSO, 6 mL of ES-FBS and 3mL alveolar media. This medium should be made fresh just before use.)
- TryplE-stopping medium (For 50 mL, add 5 mL of FBS to 45 mL of DMEM. This medium can be stored at 4 ℃ for up to 1 month.)
- MACs buffer (For 150mL, add 140mL DPBS, 10mL 0.75% BSA(see reagent-8) and 600µL 0.5M EDTA)
- Transwell (24 well, 0.4µm transparent, Greiner Bio-One, cat. no. 662641)

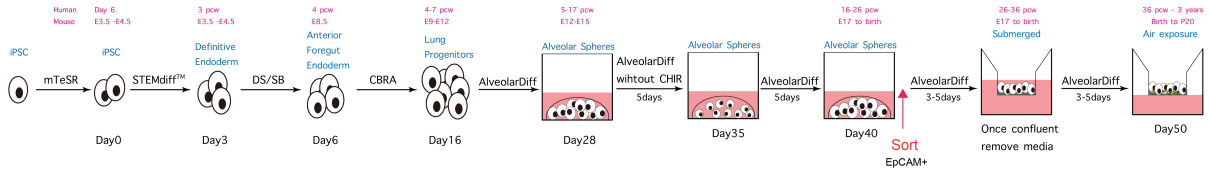

Figure 1: Overview of PSC-derived alveolosphere differentiation protocol.

3 Procedure

3.1 iPSCs preparation (∼5days, 2wells)

1. Thaw iPSCs
   - Coated 2 wells of 6-well-plate with geltrex (1:100, 100µL + 10 mL Hams/F12) for an hour
   - Prepare 4mL mTeSR1 medium with 100x Rock inhibitor at 37℃
   - Thaw 1 tube of iPSC in incubator for 2min[1]
   - Transfer cells to 15ml Falcon and add 10ml DPBS with Rock inhibitor
   - Spin cells in centrifuge. 400g for 5 minutes
   - Use automatic suction to remove DPBS
   - Add 2 mL mTeSR media with Rock inhibitor
   - Re-suspend cells by drawing falcon tube back and forth along metal grating of hood
   - Plate 75% cells in first well, 25% in second well for backup and add last media
   - Once placed the plate in the incubator, slide plate up and down 3 times, pause for 5 seconds, then slide it from left to right 3 times
   - Feed cells without Rock inhibitor every day
   - When cells are at 70–80% confluence, move to Step 2.
2. Passage iPSCs
   - Preparation.
     - Coated 2 wells of 6-well-plate with geltrex (1:100, 100µL + 10 mL Hams/F12) for an hour
     - 4mL mTeSR1 medium with 100x Rock inhibitor at 37℃
     - 12mL DPBS with Rock inhibitor
   - Aspirate mTeSR1 media.
   - Add 1 mL of TryplE at 37℃ for 2min.
   - When cells are detached, large holes will be visible in cell colonies, transfer cells to new falcons.
   - Add 6 mL of DPBS with Ri into falcons.
   - Count cells by Trypan blue.[2]
   - 400g for 5 minutes.
   - Aspirate supernatant .
   - Add mTeSR media, make cells at 1M/mL.[3]
   - Plate cells to 2 wells.[4]
   - Once placed the plate in the incubator, slide plate up and down 3 times, pause for 5 seconds, then slide it from left to right 3 times.
   - Incubate for around 5days. Feed cells every day. (Fig.2)
   - Move to Step 3 when cells are over 90% confluent.

[1] Avoid using water baths.
[2] Count cells in any method
[3] 2M cells in one well
[4] Store 1M cells for RNA

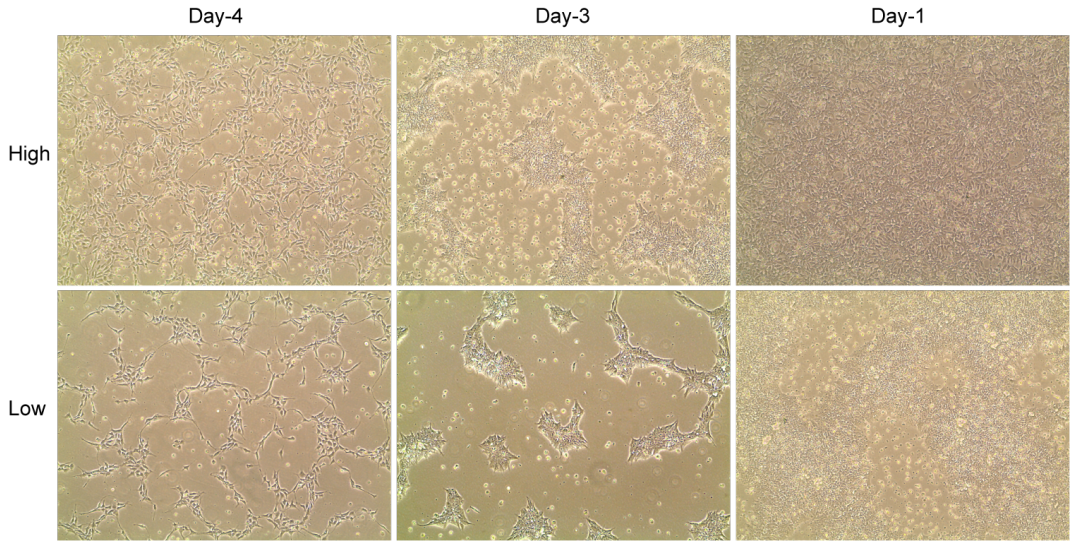

Figure 2: iPSCs preparation

3.2 Definitive endoderm induction (3days, 2wells)

3. Feed cells. Day 0
   - Preparation.
     - Thaw MR+CJ StemDiff definitive endoderm medium overnight at 4℃
     - 10mL MR+CJ StemDiff definitive endoderm medium for Day0
     - 10mL CJ-only StemDiff definitive endoderm medium for Day1
     - 10mL CJ-only StemDiff definitive endoderm medium for Day2
   - Aspirate media and add MR+CJ StemDiff medium to each well.
   - Incubate for 24h.
4. Feed cells. Day 1
   - Aspirate media
   - Add CJ-only StemDiff medium to each well.
   - Incubate for 24h.
5. Feed cells. Day 2
   - Aspirate media.
   - Add CJ-only StemDiff medium to each well.
   - Incubate for 24h, move to Step6. (Fig.3)

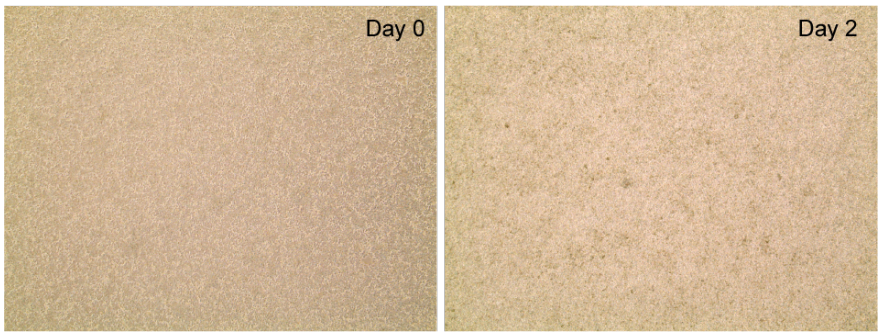

Figure 3: Definitive endoderm induction.

3.3 Anterior foregut endoderm induction (3days, 4wells)

6. Passage AFE cells -AFE[5] Day 3-5
   - Prepare 24mL DS/SB medium.
   - Passage DE cells. See Step2.
   - Plate 4 wells at 2M cells per 6wp well. Feed DS/SB medium with Rock inhibitor.
   - Store 1M definitive endoderm cells for RNA.
   - Feed cells without Rock inhibitor every day. (Fig.4)
   - At day6, store 1 well for AFE RNA and move to Step7

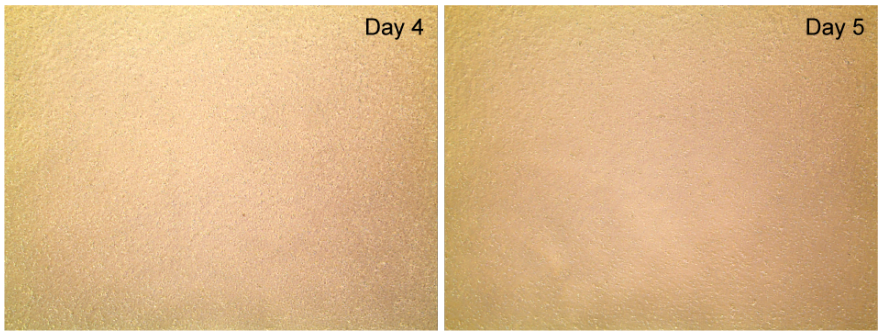

Figure 4: Anterior foregut endoderm induction.

3.4 Lung progenitor induction (8days, 3wells)

7. Feed cells. Day 6-13
   - Prepare 24mL CBRa medium.
   - Aspirate media and add warm CBRa medium to each well.
   - Feed cells every other day. (Fig.5)

[5]In Kotton lab protocol, Perform FACS analysis for expression of CKIT and CXCR4 before AFE induction. More than 80 percent of cells should co-express CKIT and CXCR4 at this stage.

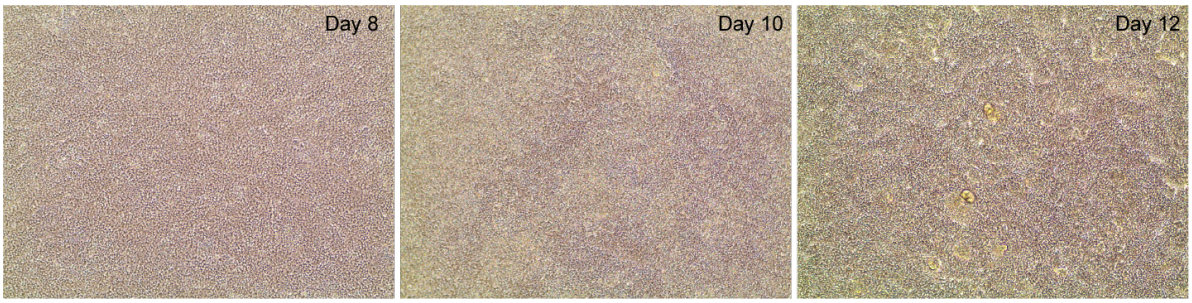

Figure 5: Lung progenitor induction.

3.5 Alveolar spheres induction (∼33days, 8wells)

8. Passage LP cells. Day 14
   - Preparation.
     - 6mL TripLE at 37℃
     - 12mL cold TripLE-stopping medium with Rock inhibitor
   - Aspirate the CBRa medium from each well.
   - Add 1 mL of warm TriplE into each well.
   - Use pipette to scratch the cell monolayer in a crosshatch pattern[6]
   - Incubate at 37℃ for 5min.
   - Pipette two to three times with a P1000 pipette. If single cells are observed, keep going. Otherwise incubate for an additional 5 min.
   - Add 2mL TripLE-stopping medium, mix gently.
   - Count cells.
   - Centrifuge cells at 400g 4℃ for 5 min.
   - Aspirate supernatant.
   - Store 1M cells for RNA.
   - Move to Step9 immediately.
9. Plate cells in geltrex. Day 14
   - Preparation
     - 6mL CK+DCI medium with Rock inhibitor at 37℃
     - Thaw 1.5mL 3D Matrigel in ice overnight[7]
   - Passage cells (Step8) or dispose gel (Step10).[8]
   - Resuspend 2000 cells/μL of undiluted 3D Matrigel[9]
   - Plate alveolospheres in 50μL Matrigel droplets in 6wp for ideally 3 wells.[10]

[6]From Kotton lab: Crosshatching of the cell monolayer should allow for increased penetration of trypsin–EDTA and for more effective enzymatic dissociation. This process will yield substantial amounts of floating cells.

[7]leave in cold room

[8]In Kotton lab, they sorted NKX2-1$^+$ lung progenitors cells at this step, but not in James lab.

[9]From Kotton lab: Matrigel can be quite difficult to work with because it rapidly solidifies at room temperature. For cells to be successfully resuspended in Matrigel, always leave 3D Matrigel on ice before and during use, and consider either freezing pipette tips before Matrigel use or coating the inside of the pipette tips with cold PBS before pipetting the Matrigel.

[10]Normally 8 droplets in each well

- Place droplets in a 37℃ incubator for 20min without media[11]
- Add the 2mL of warm CK+DCI+Rock inhibitor medium to each well
- Feed cells without Rock inhibitor every other day. (Fig.6)
- When cells are ready to passage after around 16 days[12], move to Step11.

10. Dispose gel. Day 31
    - Preparation
      - 9mL 2mg/mL Dispase with Rock inhibitor at 37℃
      - 3mL TripLE at 37℃
      - 21mL F12 with Rock inhibitor
      - Thaw 3D Matrigel in ice overnight[13]
    - Aspirate the media from each well
    - Add 1 mL of warm Dispase at 37℃ for 15min
    - Pipette to lift geldrop and add 1 mL of warm Dispase at 37℃ for 15min
    - Pipette up and down two times and add 1 mL of warm Dispase at 37℃ for 30min[14]
    - Transfer 3mL dissociated organoids in dispase to a 15mL falcon.[15]
    - Add 7mL F12 to 15mL falcons.
    - Centrifuge cells at 4℃, 200g for 5 min.[16]
    - Aspirate the supernatant.
    - Add 1mL warm TripLE. Pipette up and down two times and incubate at 37℃ for 2-3min.[17]
    - Check cells under microscope. If there are visible organoids, incubate for another 5min.[18]
    - Add 2mL TripLE-stopping medium.
    - Count cells.[19]

[11] Allow the 3D Matrigel to solidify
[12] It depends on the cell state and density.
[13] leave in cold room
[14] Make sure there is no visible gel. If so, incubate for anther 10min
[15] Or all of the cells to 50mL falcon
[16] Only organoids can be centrifuged down at this speed.
[17] No longer than 5min. Add DNase if necessary
[18] If the organoids still can be found after 10min incubation, add 2mL TripLE-stopping medium and centrifuge cells at 400g for 5 min. Re-add TripLE for 5min
[19] Leave cells at 4℃ until move to Step11 or Step9

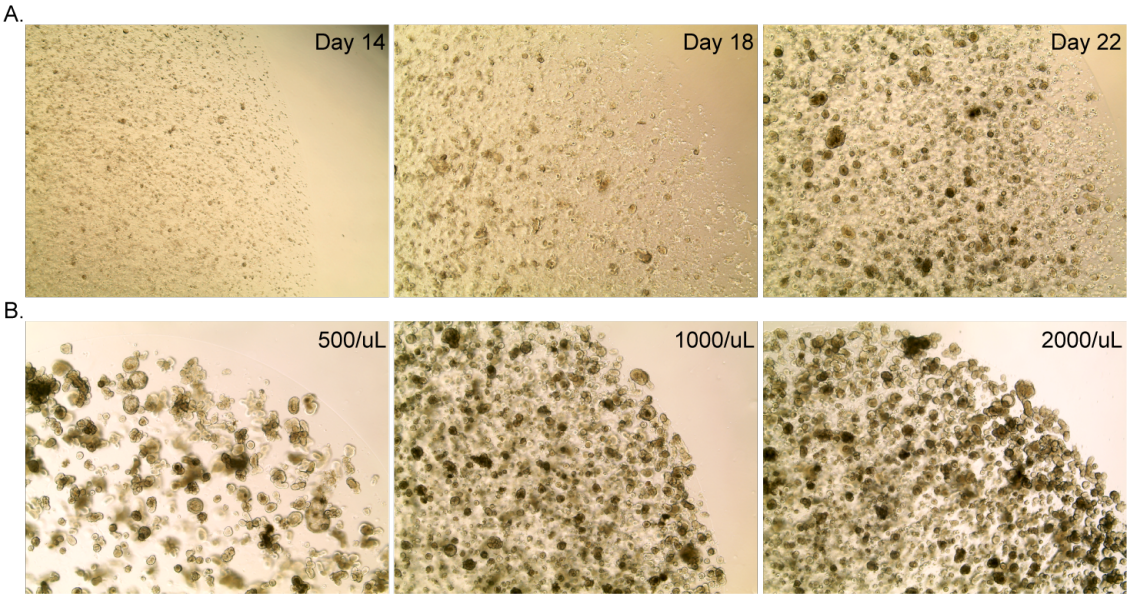

Figure 6: Alveolar spheres induction. A. 2000 cells/µL of undiluted 3D Matrigel on Day 14, 18 and 22. B. 500, 1000 and 2000 cells/µL of undiluted 3D Matrigel on Day 26.

11. Beadsort.[20] Day 31
    - Preparation
      - 20mL CK+DCI medium with Rock inhibitor at 37℃
      - 25mL MACs buffer at 4℃
      - 30mL DPBS with Rock inhibitor
      - 100uL EpCAM beads
    - Centrifuge cells at 400g for 5 min
    - Aspirate the supernatant.
    - Resuspend in 300uL MACs buffer (cold)
    - Add 100uL EpCAM beads.[21]
    - 30 mins 4 deg, mixed occasionally
    - Set up magnetic column in magnets
      - with 50mL tube under in big rack to collect runthrough
      - tilt stand forward with a rack behind
      - added 3mL MACs buffer, ran through.[22]
    - Add 5ml MACs buffer, span 400g 5min
    - Resuspend in 500uL MACs buffer, ran though
    - Add 4x 3ml MACs buffer, ran through each, added each wash straight after last run through
    - Remove column from magnet, put onto 15ml tube

[20] If researchers are first time to sort, it is recommended to seed unsorted cells in at least one well for backup.
[21] enough for 50M cells.
[22] plunger needed for final step, hard to keep sterile - best to keep in packet

- Add 5mL MACs buffer, applied plunger to push through.[23]
- Count eluted cells and also the wash-through (neg selected) cells.
- Store 1M eluted cells for RNA.
- Plated selected cells into 3D gtrex wells for ideally 8wells (see Step9).
- Incubate for 48h, then move to Step12

12. CHIR withdrawal and addback.[24] Day 33-46
    - Preparation
      - 8mL F12
      - 32mL K+DCI medium
      - 90mL CK+DCI medium
    - Remove CK+DCI medium in each well surrounding the 3D Matrigel drop
    - Wash with 1mL F12.
    - Add 2mL K+DCI medium into each well.
    - Incubate for 48h, re-feed once.[25]
    - At day37, remove K+DCI medium in each well.
    - Add 2mL CK+DCI medium into each well.
    - Incubate for 10 days, feed cells every other day by CK+DCI medium. (Fig.7)
    - When cells are ready to passage, move to Step10 or Step13.

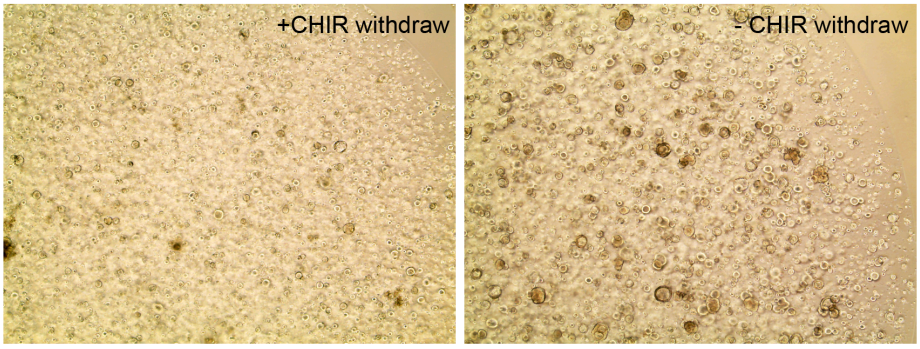

Figure 7: CHIR withdrawal and addback. 2000 cells/μL of undiluted 3D Matrigel on Day 40.

3.6 ALI induction (∼7d)

13. Plate cells in transwell. Day 47-50
    - Preparation
      - Coated 24wp transwells by 1:100 diluted geltrex.
      - 800μL CK+DCI medium with Rock inhibitor for each transwell at 37℃.
    - When cells in geltrex well are ready to passage, dispose gel (Step10).
    - Centrifuge cells at 400g for 5 min.

[23] says firmly, but don't be too firm

[24] NB: CHIR withdraw may lead to cells growing slowly. Alveolospheres cultured in K+DCI medium are much smaller than those in CK+DCI medium. So if there is no visible AT1 cells, it is recommended to skip this step and move to Step10 or Step13.

[25] Totally 4days for CHIR withdraw.

- Resuspend in 5mL DPBS.
- Count cells.
- Centrifuge cells at 400g for 5 min.
- Resuspend cells in CK+DCI medium at 50k/100μL.
- Seed 100k cells into coated 24wp transwells.
- Add 600μL media with Rock inbihitor in down wells and no more than 200μL media with Ri in top chamber.
- Incubate for around 3-5 days until monolayer is formed. Move to Step14.

14. Culture in ALI. Day 51-53
    - Prepare 700μL CK+DCI medium for each transwell at 37℃.
    - When monolayer is formed, remove media in top chambers and down wells.
    - Add 700μL alveolar medium into down wells.
    - Keep no liquid in top chamber.
    - Incubate for 3 days. Cells are ready to use. (Fig.8)

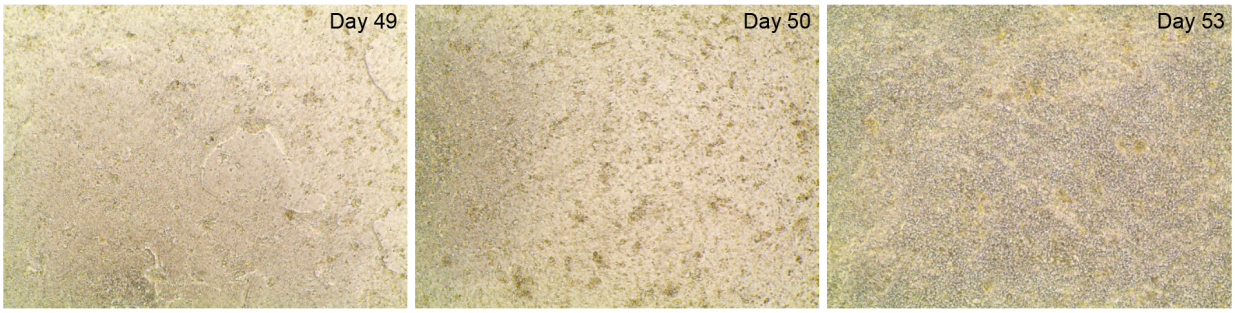

Figure 8: ALI induction. Plate cells in transwell on Day 47 and then remove the media in top chamber on Day 51.

3.7 Storage and analysis of alveolospheres

15. Freeze alveolospheres
    - Preparation
      - 1mL freeze media
      - leave cryo-safe container in -20℃ overnight
    - Dispose gel and obtain single cells (see Step10).[26]
    - Resuspend cells at a concentration of at least 1×10^6 cells in 1mL freeze media.
    - Leave the cryovial in cryo-safe container at -80℃ overnight.
    - Transfer cryo-safe container to liquid nitrogen freezer for long-term storage.
16. Alveolosphere thawing
    - Preparation
      - 12ml DPBS with Rock inhibitor
      - Matrigel
      - CK+DCI medium

[26] Alveolospheres will not survive freeze–thaw cycles if frozen as spheres rather than as single cells.

- Thaw 1 tube of alveolosphere in incubator for 2min[27]
- Transfer cells to 15ml Falcon and add 10ml DPBS with Rock inhibitor
- Centrifuge cells at 400g 15℃ for 5 min.
- Aspirate the supernatant.
- Add 2mL DPBS with Rock inhibitor.
- Re-suspend cells by drawing falcon tube back and forth along metal grating of hood.
- Count cells.
- Centrifuge cells at 400g 15℃ for 5 min.
- Re-suspend 2000 live cells/μL in undiluted 3D Matrigel.
- Plate alveolospheres in 50μl Matrigel droplets in 6wp.
- Place droplets in a 37℃ incubator for 20min without media.
- Add the 2mL of warm CK+DCI+Rock inhibitor medium to each well.
- Feed cells without Rock inhibitor every other day.

17. Analysis

- Mature iAEC2s should express many genes and contain functional lamellar bodies and produce and secrete surfactant.
  - NKX2-1
  - SFTPC
  - SFTPB
  - genes related to surfactant synthesi
    - ATP-binding cassette subfamily A member 3 (ABCA3)
    - pepsinogen C (PGC)
    - cathepsin H (CTSH)

[27] Avoid using water baths.